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Abstract

Hyperlipidemia and hyperglycemia frequently occur in obese population. As chronic, low-grade inflammation is closely associated with obesity, we investigated if polyphenol-rich blackcurrant extract (BCE) can prevent inflammation and diet-induced metabolic disturbances in mice. Male C57BL/6J mice were given a modified AIN-93M control diet containing high fat/high cholesterol (16% fat, 0.25% cholesterol by weight) or the same diet supplemented with 0.1% BCE (wt/wt) for 12 weeks. No significant differences in total body weight or liver weight occurred between the two groups. BCE-fed mice had fewer crown-like structures (CLS) with concomitant decreases in mRNA abundance of F4/80, CD68, and inhibitor of nuclear factor kB kinase e (IKKe) in the epididymal adipose tissue. F4/80 and IKKe mRNA levels were positively and significantly correlated with CLS number. BCE-fed mice demonstrated a significantly lower plasma total cholesterol (TC) and glucose levels than controls, but no significant difference in plasma triglyceride (TG) levels. BCE supplementation did not significantly alter mRNA levels of major regulators of

hepatic cholesterol metabolism, i.e., HMG-CoA reductase (HMGR) and low density lipoprotein receptor (LDLR). However, protein expression levels of mature sterol-regulatory element binding protein 2 as well as LDLR were significantly increased. In the livers of mice fed BCE, there was a significant decrease in expression of proprotein convertase subtilisin/kexin type 9 (PCSK9), which facilitates LDLR protein degradation, as well as one of its transcriptional regulators, i.e., hepatocyte nuclear factor 4 alpha. The skeletal muscle of BCE-fed mice showed a significant increase in mRNA expression of genes involved in energy expenditure and mitochondria biogenesis, including peroxisome proliferator activated receptor alpha (PPARalpha), PPARdelta, uncoupling protein 2 (UCP-2), UCP-3, and mitochondrial transcription factor A (TFAM). Upon stimulating splenocytes from BCE-fed mice with lipopolysaccharides, tumor necrosis factor alpha and interleukin-1beta mRNA levels were significantly lower than control mice (4). The results suggest that BCE supplementation decreases obesity-induced inflammation in adipose tissue and splenocytes, at least in part, by modulating energy metabolism in skeletal muscle. Beneficial effects of BCE on plasma TC and glucose, liver steatosis suggest that this berry may be consumed to prevent metabolic dysfunctions induced by diets high in fat and cholesterol.

Introduction

Obesity is a widely-noted global health concern, and in the U.S. current rates demonstrate as many as 42% of adults are projected to become obese by the year

2030 (19). It remains a leader in preventable causes of death, falling close behind tobacco smoking and elevated blood pressure (13). Chronic low-grade inflammation has been demonstrated to cause obesity-related metabolic diseases, such as insulin resistance, hyperlipidemia, hyperglycemia, type 2 diabetes, cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD) (67). However, research has uncovered that a subset of obese persons can be metabolically healthy, which decreases the risk of secondary pathogenic obesity-associated diseases (83). This indicates that obesity alone may not result in metabolic dysfunction, but rather contributes to the progression of pathogenesis for secondary diseases.

Metabolic stress resulting from fat overload, and subsequent recruitment of monocytes to adipose tissue is the cause of chronic local inflammation (27). This occurs by inducing the production of pro-inflammatory mediators in lipid-laden adipose tissue (3). Pharmaceuticals such as non-steroidal anti-inflammatory drugs are common treatments for acute and chronic inflammatory conditions, however they do come with adverse effects (32). In addition, the physiological characteristics of obesity-induced chronic inflammation differ from other inflammatory disorders such as arthritis and ulcerative colitis (37, 86). Therefore, identification of food components with anti-inflammatory properties and minimal side effects are critically needed. This will contribute to creating sufficient dietary strategies to prevent obesity-associated metabolic disorders.

Epidemiological studies have indicated that diets containing high levels of fruits and vegetables are inversely associated with the pathogenesis of obesity and CVD (81). These beneficial effects are associated with the high polyphenol content of

fruits and vegetables (56). Of the various polyphenols found in fruits, anthocyanins are the most abundant (28) and found in a wide array of berries such as blueberries, cranberries, raspberries, blackberries, chokeberries, and acai berries (87). Berries are of particular interest due to their purported health benefits, which are largely correlated with their high polyphenol content (6, 14, 18, 66, 82, 88). These benefits have been attributed to preventing cancer, diabetes, and other inflammatory diseases (24, 75, 88). Though blueberry, cranberry, blackberry, and raspberry are commonly consumed in the U.S., blackcurrant has recently increased in popularity. Blackcurrant is rich in anthocyanins and vitamin C (74), and is known to hold a higher antioxidant capacity compared to other commonly consumed berries (46, 51, 84).

Studies have demonstrated that blackcurrant exhibits anti-inflammatory, antioxidant, and anti-microbial effects, which could provide potential health benefits against hypertension, CVD, neurodegenerative disease, ocular diseases, and hypercholesterolemia (20, 25, 30, 35, 40, 74, 78, 79). Consumption of blackcurrant has also been shown to improve insulin sensitivity and inhibit inflammation (45). However, the mechanisms of action for the effects of blackcurrant on obesity-associated inflammation have never been investigated. The goal of this study was to determine the possible roles and mechanisms of polyphenol-rich blackcurrant extract (BCE) in preventing obesity-associated metabolic abnormalities in mice fed a diet high in fat and cholesterol (4).

Materials and Methods

Animal care and diet

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) at 15 weeks of age were randomly assigned into a control or BCE group. The control group of 11 mice received a modified AIN-93M containing high fat/high cholesterol diet (HF/HC; 16% fat, 0.25% cholesterol by weight; 55.7%, 125.5%, and 31.8% energy from carbohydrate, protein, and fat, respectively; 4529 kcal/g). The BCE group was fed a HF/HC diet supplemented with 0.1% of BCE by weight. Artemis International, Inc graciously supplied the standardized BCE powder containing 25% anthocyanins and 40% polyphenols. Based on body surface normalization to a 70 kg individual (62), 0.1% BCE containing 25% anthocyanins is equivalent to daily consumption of ~540 mg BCE and 135 mg anthocyanins in humans. As the average daily intake of anthocyanins per person has been estimated to be ~200 mg in the U.S. (45), we believe the dietary level of berry extracts is attainable in humans (4). While developing HF-induced obesity, mice were given BCE to determine its preventative effects on obesity and its associated dysfunctions. Housing for mice was maintained in a controlled environment with 12h light/dark cycles and ad libitum feeding during the course of the study. Weekly body weight and food consumption were recorded along with monthly blood draws from the lateral tail vein. Post 12 weeks on the experimental diets, mice were fasted for 8 h and anaesthetized by injecting ketamine/xylazine (100/10 mpk) (Henry Schein Animal Health, Dublin, OH) (4). Cardiac puncture was used to collect ~1mL blood samples into a BD vacutainer containing EDTA, and mice were sacrificed by exsanguination followed by cervical

dislocation. Epididymal and retroperitoneal fat pads were harvested, weighed, and snap frozen in liquid nitrogen for later use in gene analysis, or were fixed in 10% formalin for histological analysis. Gastrocnemius muscle samples were also collected, snap frozen in liquid nitrogen, and stored at -80^{o}C for gene analysis (4). Blood was centrifuged at 1,500xg for 10 min at 4^{o}C. Livers were weighed, and subsamples were snap frozen in liquid nitrogen and stored at 80^{o}C until use or fixed in 10% formalin (4). The Institutional Animal Care and Use Committee of the University of Connecticut approved all animal procedures.

Gene expression analysis of liver and muscle by quantitative realtime PCR (qRT-PCR)

Total RNA extraction was performed on liver samples, epididymal fats, and muscle tissue using TRIzol reagent (Invitrogen, Grand Island, NY). The expression of genes involved in fat, cholesterol, and glucose metabolism was measured using qRT-PCR analysis. This was accomplished using the SYBR Green procedure and CFX96 realtime PCR detection system (BioRad, Hercules, CA) (40, 41, 42, 43, 59, 85, 86). Primer sequences were designed according to GenBank database using the Beacon Designer software (Premier Biosoft, Palo Alto, CA). Ribosomal protein large P0 (RPLP0) and Beta-actin represent the internal controls, and were used in calculating $2^{-\Delta\Delta Ct}$. These values were used to ascertain the validity of the chosen internal control for the analysis. Using each internal control, the data analyzed demonstrated a similar trend of changes in gene expression and the data reported in this study used RPLP0 as an internal control.

Western blot analysis

Western blot analysis was performed with liver lysates (61). Antibodies used were: low-density lipoprotein receptor (LDLR; Abcam, Cambridge, MA), HMG-CoA reductase (HMGR; Santa Cruz Biotechnology, Dallas, TX), mature sterol-regulatory element binding protein 2 (mSREBP-2; Abcam) and beta-actin (Sigma, St. Louis, MO) (4). Blots were developed using horseradish peroxidase (Thermo Fisher Scientific, Rockford, IL) and densitometry analysis was performed using Chemidoc XRS+ (Bio-Rad) and Image Lab software (Bio-Rad). Beta-actin was used as a loading control (4).

Statistical Analysis

Student's conducted t-tests and results were used with the GraphPad Instat 6 (GraphPad Software, La Jolla, CA) to compare mean differences between groups. All data are expressed as means $\pm$ SEM, and an alpha-level of $P<0.05$ was considered statistically significant (4).

Results

Reduced expression of lipogenic genes in hepatic tissue by BCE supplementation

Measuring the hepatic expression of LDLR and HMGR, and mSREBP-2, a transcriptional regulator of LDLR and HMGR, provided mechanistic insight into the total cholesterol-lowering effects of BCE. The abundance of mRNA for LDLR and HMGR did not indicate a statistically significant difference between control and BCE groups (Figure 1A). However, Western blot analysis did indicate significantly higher

protein levels of mSREBP-2 and LDLR in mice fed BCE-diets than controls, but showed no difference in HMGR protein levels (Figure 1B and C). mRNA expression of fatty acid oxidation genes, carnitine palmitoyltransferase 1α (CPT-1α), CPT-1β, and acylCoA oxidase 1 (ACOX-1), were not significantly altered by supplementation with BCE (Figure 2). Hepatic expression of gluconeogenic genes, as in phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphate (G6P), showed no significant difference for both groups.

Alterations in the expression of metabolic genes in the muscle by BCE supplementation

Although the percentage of epididymal fat weight was significantly reduced in the BCE-fed mice, there were no significant differences in the expression of genes involved in lipid synthesis and fatty acid oxidation, such as SREBP-1c, PPARγ, fatty acid synthase (FAS), stearoyl CoA desaturase 1 (SCD-1), CPT-1α and 1β, in the epididymal fat (data not shown).

To investigate the potential effect of BCE supplementation on energy metabolism in muscle, which would contribute to potential alterations in total body weight, we measured expression of genes involved in fatty acid beta oxidation and mitochondrial uncoupling/biogenesis. PPARα, PPARδ, UCP-2, UCP-3, and TFAM expression demonstrated significant increases in the muscle of BCE-fed mice compared to the control group (Figure 3, 4, and 5). In addition, BCE supplementation showed a trend toward increased in expression of ACOX-1 and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α).

Discussion

Obesity significantly increases the risk of CVD, NAFLD, diabetes mellitus, hypertension, arthritis, asthma, and cancer, which remain major health problems in the U.S. (71). Chronic, low-grade inflammation is a common factor associated with the pathogenesis of obesity-related metabolic diseases (71). Identifying dietary products that lower blood lipids and glucose would contribute to lowering the disease risk. Many natural food products having anti-inflammatory properties have been identified, though novel discoveries would contribute to the list of potentially therapeutic dietary components (3). Blackcurrant cultivation doesn't have a substantial history in the U.S., however its potential health benefits have granted it a significant amount of attention. Blackcurrant has been demonstrated to have a myriad of therapeutic properties, acting as an antioxidant, anti-inflammatory, and anti-microbial agent [25]. However, the role of blackcurrant in modulating obesity-induced inflammatory conditions has yet to be investigated (3). This study shows that BCE supplementation lowered plasma TC and decreased fat accumulation in the liver and plasma glucose, without altering the expression of genes involved in lipogenesis, fatty acid oxidation, or gluconeogenesis. We presume that the prevention of hepatic steatosis and hyperglycemia by BCE supplementation is secondary to its effects on extra-hepatic tissues, such as skeletal muscle.

Skeletal muscle is a metabolically active tissue that contributes largely to energy expenditure in the body. Due to the absence of change in regard to lipogenic and fatty acid oxidative gene expression in adipose tissue by BCE supplementation, despite less macrophage infiltration, we investigated the effect of BCE on lipid

metabolism and mitochondrial biogenesis in skeletal muscle. In mice fed a BCE-diet compared to controls, fatty acid beta-oxidation genes, i.e. CPT-1α, CPT-1β, and ACOX-1, showed no significant difference coupled with an increasing trend for ACOX-1 (P = 0.08). Although, an observed significant induction of PPARβ and PPARδ by BCE supplementation in muscle with an associated increase in UCP-2 and UCP-3 expression was noted. The PPAR genes have been demonstrated to act as transcriptional regulators for UCP-2 and UCP-3 expression in skeletal muscle tissue [80]. UCP1 functions mainly in brown adipose tissue, while UCP2 and UCP3 are expressed in other tissues including skeletal muscle [57]. UCP-2 and UCP-3 act by uncoupling respiration from ATP synthesis in the mitochondria, which facilitates the dissipation of energy as heat [34] and prevents buildup of reactive oxygen species [44, 55]. UCP-2 and UCP-3 have also been shown to play important roles in glucose and lipid metabolism [15, 33]. As an example, UCP-3 overexpression in skeletal muscle of mice was shown to protect against obesity by lowering fasting plasma glucose and insulin [10]. Concurrent with this observation, we also saw a $\sim$35% reduction in fasting plasma glucose for the BCE group (data not shown). In addition, we also saw a significant increase in TFAM expression coupled with an increasing trend in PGC-1α, a large component in mitochondrial biogenesis and uncoupling [17], in the skeletal muscle of BCE mice. Further investigation is necessary to address if BCE enhances mitochondria biogenesis and energy expenditure in skeletal muscle; however, our observations strongly suggest that BCE may exercise its anti-inflammatory properties in adipose tissue by modulating energy metabolism in skeletal muscle (3).

Achieving the preventative and therapeutic goal to lower circulating cholesterol is accomplished by the induction of LDLR expression and activity in the liver. Prescribed cholesterol-lowering drugs, such as statins, inhibit HMGR activity which in turn increases LDLR expression [26]. Inducing LDLR expression mainly depends on SREBP-2, a widely recognized transcriptional regulator of LDLR, which also acts by upregulating HMGR expression [69]. High levels of cholesterol in the cell cause insulin-induced genes (INSIG) to bind to SREBP-2 in complex with SREBP cleavage-activating protein (SCAP) in the endoplasmic reticulum. This prevents the translocation of the SREBP-2/SCAP complex to the Golgi [31]. Lowered levels of cellular cholesterol cause the SREBP-2/SCAP complex to be released from INSIG and transported to the Golgi, where it undergoes two step proteolytic cleavage, releasing the N-terminal transcriptional activation domain, mSREBP-2, which in turn induces LDLR and HMGR transcription. This study demonstrates that despite a substantial increase in mSREBP-2 protein levels in the livers of BCE-fed mice, LDLR and HMGR mRNA levels were not significantly altered. LDLR protein levels in the liver, however, did increase by ~80% for the BCE group compared to the control group. The result indicates that an increase LDLR protein is likely due to effects of BCE at a post-transcriptional level.

Mice fed BCE, when compared to controls, showed attenuated liver steatosis by histological analysis. We measured the expression of genes involved in lipogenesis and fatty acid oxidation to investigate the mechanisms of action. BCE supplementation was shown to significantly lower FAS mRNA in the liver, however its protein levels revealed no difference between groups (data not shown). In

addition, a trend toward a decrease rather than increase for expression of genes related to mitochondrial fatty acid oxidation, as in CPT-1α and 1β, was demonstrated in the livers of BCE-fed mice. BCE supplementation did not alter mRNA expression of ACOX-1, which is an important enzyme for peroxisomal fatty acid oxidation. This indicates that the mechanism of action for BCE in inhibiting the development of liver steatosis is not likely attributed to lipogenesis or fatty acid oxidation (3). We are interested in the findings of our recent report indicating that genes related to energy expenditure and mitochondrial biogenesis in the skeletal muscle of BCE fed mice were significantly increased, including PPARα, PPARδ, UCP-2, UCP-3, and TFAM [3]. No significant changes were detected in expression of genes for lipid metabolism in the adipose tissue. These findings support the notion a decrease in liver steatosis may prove secondary to the effects of BCE on energy metabolism in the skeletal muscle. BCE supplementation also significantly decreased plasma fasting glucose levels by ~35%, but did not significantly alter hepatic expression of gluconeogenic genes, i.e., in G6P and PEPCK. UCP-2 and UCP-3 are known to play crucial roles in glucose and lipid metabolism [15, 33], and overexpression of UCP-3 in skeletal muscle shows lowered fasting plasma glucose and insulin [10]. Therefore, we propose the beneficial effects of BCE supplementation in preventing liver steatosis and hyperglycemia are likely due to enhanced energy utilization in the skeletal muscle. Further investigation should be warranted to test this possibility.

Conclusion

This study is among the first to investigate the potential health benefits of BCE in preventing obesity-induced inflammation and hyperglycemia. Although further investigation is needed for understanding the exact mechanisms of action for the beneficial effects of BCE, our study strongly suggests that BCE consumption may prevent an array of metabolic dysfunctions related to high fat and high cholesterol diet.

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Figure Legend

Figure 1. Expression of mRNA and protein levels of lipogenic genes in the livers of male C57BL/6J mice fed a HF/HC control or 0.1% (w/w) BCE supplemented diet for 12 weeks. **A**. mRNA expression. **B**. Western blot image. **C**. Protein levels (quantification). Values are means ± SEM; n= 11 for control and 13 for BCE

Figure 2. Expression levels for genes involved in fatty acid beta-oxidation between control and BCE mice; taken from skeletal muscle tissue.

Figure 3. Gene expression changes in lipid metabolism between control and BCE groups.

Figure 4. Mitochondrial biogenesis gene expression trends in skeletal muscle.

Figure 5. Changes in expression of genes involved in mitochondrial uncoupling for control and BCE groups.

Figure 2-5. Metabolic gene expression in skeletal muscle tissue taken from the gastrocnemius of male C57BL/6J mice fed a HF/HC control or 0.1% (wt/wt) BCE supplemented diet for 12 weeks. Data are expressed as relative expression to control. Values are means ± SEM; *, P<0.05; n= 11 for control and 13 for BCE

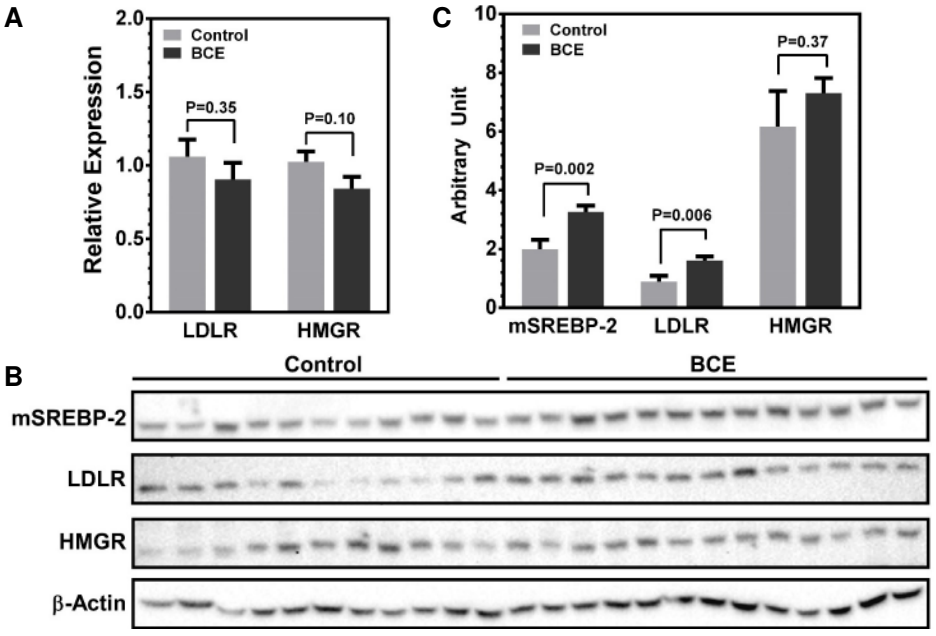
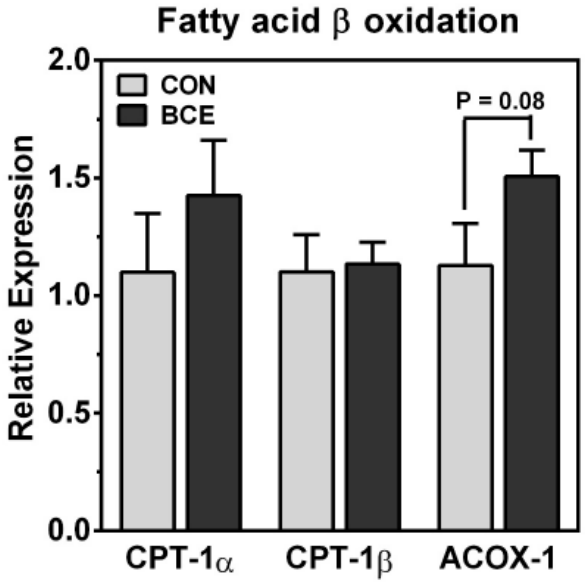


Figure 1.



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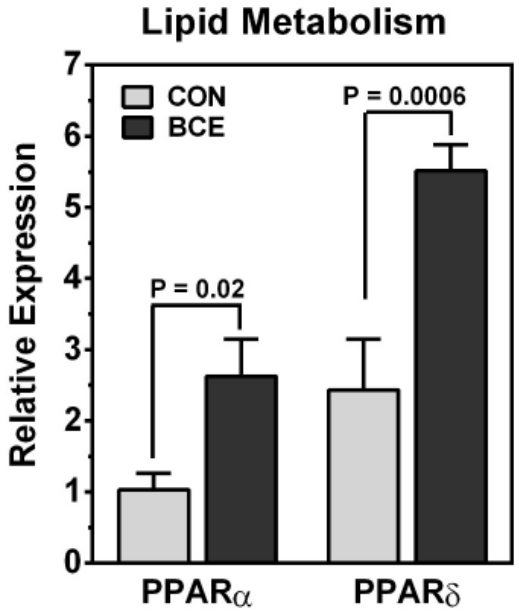

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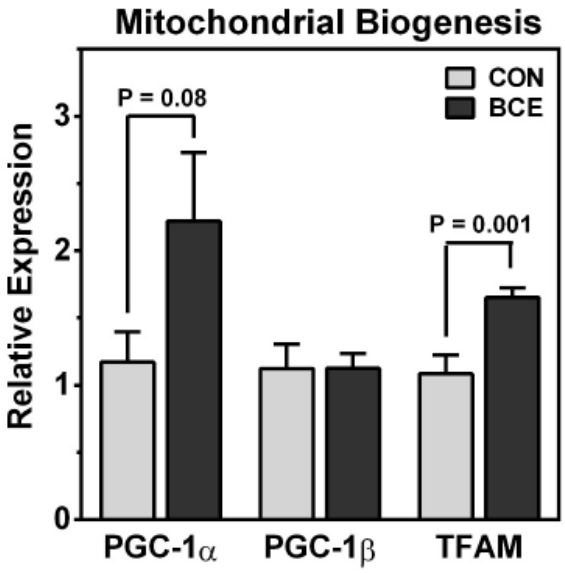

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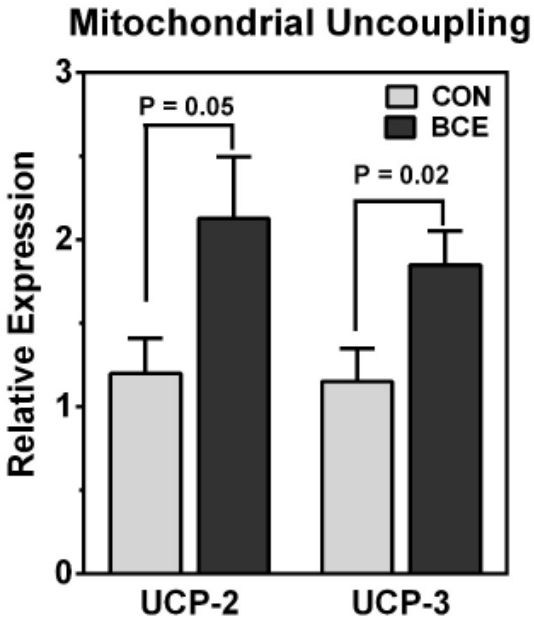

Figure 5.